

A Critical Review of Methods for Comparing Estrogenic Activity of Endogenous and Exogenous Chemicals in Human Milk and Infant Formula

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The two primary sources of nutrition for infants are human milk and infant formula. Both contain an array of endogenous and exogenous chemicals that may act through many separate hormonal mechanisms. The safety of infant nutrition sources has been questioned based on the possibility that exogenous chemicals may exert adverse effects on nursing or formula-fed infants through estrogen-mediated mechanisms. In response to these and other concerns, the National Research Council recommended assessing the estrogenic potency of natural and anthropogenic hormonally active agents. Furthermore, the Endocrine Disruptor Screening and Testing Advisory Committee of the U.S. Environmental Protection Agency specifically recommended testing chemicals present in human milk as a representative mixture to which large segments of the population are exposed. To date, no clinical or epidemiologic evidence demonstrates that levels of chemicals currently found in human milk or infant formulas cause adverse effects in infants. Nonetheless, the question is sufficiently important to warrant a consideration of how best to evaluate potential estrogenic risks. We reviewed the types of data available for measuring estrogenic potency as well as methods for estimating health risks from mixtures of chemicals in infant nutrition sources that act via estrogenic mechanisms. We conclude that the science is insufficiently developed at this time to allow a credible assessment of health risks to infants based on estimates of estrogenic potency or on an understanding of toxicologic effects mediated by estrogenic mechanisms. However, clinical and epidemiologic data for infant nutrition sources may provide insights about risks of such substances in human milk and infant formulas. *Key words:* chemical mixtures, children's health, cumulative risk, endocrine disruptors, environmental estrogen, human milk, risk assessment, toxicity equivalence factors. *Environ Health Perspect* 111:1020–1036 (2003). doi:10.1289/ehp.6023 available via <http://dx.doi.org/> [Online 25 February 2003]

In its review of hormonally active agents in the environment, the National Research Council (NRC 1999) recommended that further investigations of human exposure to natural and anthropogenic hormonally active agents be conducted to determine relative contributions of estrogen equivalents. The NRC (1999, p. 273) further recommended that the

biological potency of hormonally active agents must be related to that of endogenous hormones in premenopausal and postmenopausal women and in men. Additional comparisons should be made with pharmacologic estrogens (hormone-replacement therapy and hormonal contraceptives) and phytoestrogens because large segments of the population are exposed to these compounds.

In addition, the Endocrine Disruptor Screening and Testing Advisory Committee recommended that the U.S. Environmental Protection Agency (U.S. EPA) screen and potentially test “representative mixtures to which large . . . segments of the population are exposed,” including human milk (EDSTAC 1998), which has raised questions regarding whether appropriate methods and data are available for performing such an assessment (LaKind and Berlin 2002).

The assessment implied by the recommendations of those scientific bodies would involve using biological mechanistic information coupled with exposure data to assess overall human health risk for a particular mechanism. This concept is not new and has been used, for example, to evaluate risks to dioxin-like chemicals that are presumed or demonstrated to act through an aryl hydrocarbon (Ah) receptor-mediated mechanism (i.e., the toxic equivalents, or TEQ, approach). Similarly, Safe (1995) and NRC (1999) have compared the toxic potency of dietary and environmental estrogenic chemicals, using the concept of estrogen equivalents (EQs) where the substances are presumed to act through an estrogen receptor (ER)-mediated mechanism. On the basis of the EQ approach, Safe (1995) estimated that dietary intake of EQs from naturally occurring estrogenic compounds far exceeds dietary intake of man-made estrogenic compounds.

In an extension of this approach, we initially sought to apply methods for estimating relative estrogenic potencies of endogenous and exogenous chemicals to assessing estrogenic risks for the two primary sources of infant nutrition: human milk and infant formulas. Both types of infant nutrition are

complex mixtures of chemicals, and both contain an array of substances that have potential estrogenic activity. In combination with information on concentrations of endogenous and exogenous chemicals in these nutrition sources, estrogenic potency estimates would theoretically allow us to evaluate the relative magnitude of hormonal activity from naturally occurring substances compared with hormonal activity from exogenous substances. However, for reasons enumerated in this review, we believe that the current state of scientific understanding does not allow for accurate estimates by such methods. Although we recognize that hormonally active agents encompass a wide range of biochemical mechanisms, the focus of this paper is estrogenicity, because it is the hormonal mechanism for which most information exists.

To explain our conclusion, we first provide a review of endogenous hormones and exogenous chemicals found in human milk that have been reported to be hormonally active, particularly those that are known, suspected, or purported to act through the ER. Second, we review the phytoestrogens and other hormonally active agents found in certain infant formulas. Third, we examine the mechanisms of estrogen action and various types of *in vivo* and *in vitro* assays used to measure estrogenic potency and biologic effects. We close with a review and analysis of methods to assess the potency of estrogenic mixtures that could be used in the evaluation of human milk and formula. Because the goal of such an analysis is to predict whether hormonally active agents in infant nutritional sources are related to adverse health outcomes, we also summarize the epidemiologic and clinical literature on associations between infant exposures to hormonally active chemicals and health outcomes, and

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identify data gaps in the literature that might define future research needs. We conclude that the clinical and epidemiologic data for infant nutrition sources are currently the most reliable source of information regarding potential adverse health outcomes and provide a sufficient basis for drawing conclusions about risks to infants.

Endogenous Hormones in Human Milk

The improved ability to measure accurately a wide range of substances in human milk has led to the detection of a greater number of hormones, including both nonpeptide hormones such as thyroxine (T_4) and hormonally active peptides/proteins like prolactin and somatostatin (Koldovsky 1995; Koldovsky and Strbák 1995). Types and levels of hormones in milk have been reviewed previously (Britton and Kastin 1991; Grosvenor et al. 1992; Hamosh 2001; Koldovsky 1995; Lawrence and Lawrence 1999). Human milk contains pituitary, hypothalamic, pancreatic, thyroid, parathyroid, adrenal, gonadal, and gut hormones, sometimes in concentrations exceeding maternal plasma levels (Ebrahim 1996; Grosvenor et al. 1992). There is evidence that many hormones are absorbed by the gut of the neonate into the neonatal circulation and have important functions in the neonate (Grosvenor et al. 1992). However, whereas much is known about the function of hormones in the infant (Bernt and Walker 1999; Koldovsky 1995) (e.g., the positive influence of cortisol, an adrenal hormone, on the maturation of the immature intestinal barrier), in many cases the exact function of hormones in the infant is unknown.

Hormones reported in human milk are summarized in Table 1. Some of these hormones are transported into milk from maternal circulation unchanged in structure, others are modified, and several are not only transported into the milk but are produced within the mammary gland (Ebrahim 1996; Hamosh 2001). It has been suggested that this large number and variety of hormones establishes the mammary gland as a major endocrine organ (Ebrahim 1996).

In assessing the levels of endogenous hormones in human milk, an additional layer of complexity is added when one considers that concentrations of many hormones in human milk change over time (Hamosh 2001). For example, concentrations of estrogens in human milk vary over the course of the day and during lactation, with levels decreasing during the first 5 days postpartum and then remaining somewhat steady until 6 weeks postpartum (Grosvenor et al. 1992). Concentrations of other hormones such as insulin are highest in colostrum and decrease with progressive lactation (Britton and Kastin 1991). Another

gonadal hormone, progesterone, is present in milk in varying levels depending on the stage of reproduction, decreasing considerably 24 hr after parturition (Grosvenor et al. 1992). Milk content of hormones could also be influenced by circadian rhythms, number of infants previously breast-fed, and mammary gland from which the breast milk was sampled (Britton and Kastin 1991).

Suspected Exogenous Estrogens in Human Milk

Exogenous substances (also called environmental chemicals) may appear in human milk if the mother has been exposed, intentionally or unintentionally, through various routes such as the oral, inhalation, or dermal pathways. Environmental chemicals that are persistent and lipophilic can be globally dispersed and bioaccumulate in the food chain; exposures to these chemicals occur in most geographic locations. Other chemicals are easily metabolized and excreted or their exposures are transient, so their appearance in human tissues may be more sporadic. Environmental chemicals that are lipophilic, those that bind to milk proteins, and others in equilibrium in the body can be found in human milk. One of the first studies demonstrating that a group of environmental chemicals—chlorinated organic chemicals—was present in human milk was published in 1951 (Laug et al. 1951). The results showed that human milk may contain chlorinated organic pesticides such as DDT [trichloro-2,2-bis(*p*-chlorophenyl)ethane]. Since that study was published, many additional human milk studies have been conducted in numerous countries, and information on concentrations of environmental organic chemicals in human milk has been made available in the published literature and government documents.

Several of the chemicals detected in human milk have been reported to be estrogenic—for example, certain polychlorinated biphenyls (PCBs), DDT and its metabolites, toxaphene, dieldrin, and lindane (Jensen and Slorach 1991; NRC 1999). In addition, chlorinated dioxins and furans have been reported to exhibit estrogenic activity (Romkes and Safe 1989). Although it is outside the scope of this review to examine hormonal activity of pharmaceuticals, clearly many of these, including contraceptives, have the potential to influence the overall hormonal activity of breast milk; for example, there have been reports of feminization of male infants breast-fed by mothers using oral contraceptives (Grosvenor et al. 1992). It should also be noted that breast-feeding mothers consuming diets with soy foods have higher isoflavone levels (conjugate and free) in their milk; it has been postulated that this may be related to a lifetime protective effect against cancer for the breast-fed population (Franke and Custer 1996).

Because the data describing levels of environmental chemicals in breast milk from women residing in the United States are geographically limited and generally from small populations (LaKind et al. 2001), it is likely that human milk monitoring will be an integral part of the planned National Children's Study in the United States, as well as in other smaller-scale studies in the United States. As this body of literature grows and methods for collecting the information are coordinated (LaKind and Berlin 2002), an improved description of environmental chemicals suspected to be hormonally active in the breast milk of women residing in the United States will emerge.

Suspected Exogenous Estrogens in Infant Formulas

Soy-based formulas account for approximately 10–20% of infant formulas purchased in the United States (Essex 1996), with an estimated 750,000 infants fed soy-based formulas each year (Strom et al. 2001). Infants may have greater exposure to phytoestrogens than at any other life stage (Whitten and Naftolin 1998). Soy-based formulas are manufactured from soy protein isolates (Setchell et al. 1998) and contain the isoflavone phytoestrogens genistein and daidzein, with isoflavone levels ranging from mean values of 32 mg/L to 47 mg/L (corresponding to an intake of approximately 6–9 mg/kg/day for an infant fed soy formula) (Setchell et al. 1998; Whitten and Naftolin 1998). This is compared with approximately 6 µg/L isoflavones in human milk, or approximately 4 µg/kg/day for a breast-feeding infant (Setchell et al. 1998; Whitten and Naftolin 1998), although levels in human milk may increase an order of magnitude when the

Table 1. Some hormones and their concentrations found in human milk.^a

Hormone	Concentration
Pituitary	
Prolactin	20–90 ng/mL
Growth hormone	5–30 µU/mL
Thyroid-stimulating hormone	2.7–5.0 µU/mL
Hypothalamus	
Thyroid-releasing hormone	0.025–1.5 ng/mL
Luteinizing hormone–releasing hormone	Not available
Somatostatin	23–113 pg/mL
Gonadotropin-releasing hormone	0.1–4.0 ng/mL
Growth hormone–releasing hormone	23–430 pg/mL
Thyroid	
Thyroxine (T_4)	0.3–12 ng/mL
Triiodothyronine (T_3)	0.2–0.4 ng/mL
Reverse T_3	0.008–0.15 ng/mL
Parathyroid	
Parathormone	15 pg/mL
Parathormone-related peptide	30–50 ng/mL
Calcitonin/colatonin inhibiting protein	0–5 ng/mL
Steroid	
Estrogen	15–840 ng/mL
Progesterone	10–40 ng/mL
Adrenal steroids	0.2–32 ng/mL

^aData from Hamosh (2001).

lactating mother consumes soy food (Setchell 1996). Infants experience a high steady-state plasma concentration of isoflavones because of reduced intestinal biotransformation and from daily exposure through feeding (Setchell et al. 1998).

In addition to hormonally active agents found in soy-based formulas, other agents may be found in the water used to prepare the formula; for example, chemicals found in streams in the United States include surfactants, pesticides, plasticizers, hormones, pharmaceuticals, and components of personal care products (Barnes et al. 2000; Kolpin et al. 2002). In addition, there has been some controversy surrounding the assertion that bisphenol A, which is used to make polycarbonate baby bottles, can leach into the formula or human milk in the bottle (Raloff 1999).

Assessing Estrogenic Potency

The above review of hormonally active agents in human milk and formula clearly documents that regardless of the source of infant nutrition, infants are likely to be exposed to mixtures of endogenous and/or exogenous chemicals, some of which may be hormonally active at sufficient doses. At issue is whether exogenous chemicals contribute significantly to the estrogenicity of these chemical mixtures from a toxicologic or clinical standpoint. The question of estrogenic contribution focuses on a particular mechanism of action by which chemicals may produce toxicity (e.g., mediated by the ER) rather than on a toxic effect *per se* (i.e., mediated by any one of a variety of possible mechanisms or nonspecifically), as is typically evaluated by human health risk assessment methods described in guidance from the National Research Council (NRC 1983) and the U.S. EPA (U.S. EPA 1989, 2000), the dioxin TEQ approach notwithstanding. Toxic potency is typically evaluated from the lowest dose that produces adverse systemic effects or from the slope of the tumor dose–response curve. Risk is evaluated from a measure of exposure (or dose) and the estimate of toxic potency.

This focus on mechanism is evident in the NRC recommendation to compare estrogen equivalents for exogenous chemicals and chemicals found naturally in foods (NRC 1999). Comparing estrogenic equivalents as a surrogate for adverse effects presents a considerable challenge to toxicologists because few assays, if any, simultaneously provide information about the dose–response relationship for adverse effects and about the mechanism by which those effects are produced. The use of biological mechanistic information to evaluate the potential for adverse effects has been put forth as a useful methodology for groups of chemicals whose toxicity is assumed to be produced through a clearly defined mechanism.

Before discussing the application of mechanistic approaches to evaluate estrogenic potency, a very brief overview of estrogenic action is in order. In a classical sense, the term “estrogenic” refers to the ability of a chemical to mimic a principal *in vivo* action of the hormone estradiol, such as stimulating cornification of the vaginal epithelium, evoking estrus behavior or “heat” (the receptivity of a female animal for a male), or inducing uterine enlargement (Baird et al. 1995; Lieberman 1996; NRC 1999). In most situations, estradiol binds to specific ERs in estrogen-sensitive target cells where the hormone-liganded receptor complex, on interacting with the genome, induces transcription of specific genes and expression of estrogen-inducible proteins. The ER is a member of the steroid receptor family, a discreet subgroup of the nuclear receptor superfamily expressed in vertebrates that includes the estrogen, androgen, progesterone, glucocorticoid, and mineralocorticoid receptors (Baker 1997; Escriva et al. 1997; Laudet 1997; Mangelsdorf et al. 1995; Thornton 2001). The structure and function of nuclear receptors (Beato et al. 1996; Mangelsdorf et al. 1995; Tsai and O'Malley 1994; Weatherman et al. 1999) and mechanisms of transcriptional modulation by ERs (McKenna et al. 1999a, 1999b; Moras and Gronemeyer 1998) has been reviewed elsewhere.

Recently it has been discovered that there are at least two isoforms of the ER: ER- α and ER- β . The available data indicate that these isoforms are differentially distributed throughout the body, differ developmentally, and exhibit distinct functions (Chang and Prins 1999; Gustafsson 2000; Kuiper et al. 1996). These molecular events lead to cell proliferation and hypertrophy, seen physiologically as increased mass of estrogen-sensitive organs including the uterus and vaginal epithelium (Baird et al. 1995; Lieberman 1996; National Academy of Sciences 1999). The increasing need to identify large numbers of potentially estrogenic compounds has led to the development of a variety of *in vitro* assays based on steps within or in close proximity to mechanisms of estrogen action, such as binding to the ER or expression of estrogen-responsive genes. For reasons only partially explained to date, chemicals that interact with the ER-signaling pathway can either mimic or antagonize the actions of estradiol. Hence, such endocrine-active chemicals—called environmental estrogens, estrogen mimics, or xenoestrogens—may mimic or antagonize the action of estradiol, depending on the chemical in question and target cell examined (Witorsch 2000, 2002).

To apply a mechanistic approach to evaluate the estrogenic potency of infant nutrition sources, a number of assumptions must be made. Those assumptions include *a*) that hormonal mechanisms can be discreetly categorized

(e.g., the substance in question binds to the ER); *b*) that mechanistic measures of hormonal potency are directly related to biologic potency and activity (i.e., that the binding affinity relative to a standard is equivalent to the potency of the substance relative to the same standard in producing a biologic effect, such as proliferation of cultured target cells or uterine enlargement); and *c*) that mechanistic measures of hormonal potency can be used to predict hormonal effects of chemical mixtures. When comparing potencies is intended to evaluate potential health risks, a fourth assumption must be added: *d*) that the potential for adverse effects is directly related to mechanistic measures of hormonal potency. The validity of these four assumptions will determine, to a large extent, how reliably one can compare the potential for chemicals from various infant nutrition sources to produce adverse effects via an estrogenic mechanism. To evaluate the feasibility of applying a mechanistic approach to any particular set of chemicals, one must assess the validity of the assumptions against the existing data on endogenous hormones and the chemicals of interest.

The potential for a chemical to exhibit estrogenic activity at the molecular (or mechanistic) level can be measured by several different assays *in vitro*, including receptor binding or competitive ligand displacement assays (usually involving disrupted cells or cell extracts) and recombinant receptor-reporter gene assays (cells transfected with an ER-reporter gene construct), often called transcription activation assays. Estrogenicity of chemicals has been estimated *in vitro* using cultures of estrogen responsive cells (i.e., those already possessing ERs) such as MCF-7 (the “E-screen” assay) or T-47-D human breast cancer cells. In these systems, the end point has been either expression of specific estrogen-responsive genes or cell proliferation. *In vivo*, molecular responses can be measured as gene expression in estrogen-responsive tissues (Andersen et al. 1999; EDSTAC 1998; Witorsch 2000, 2002; Zacharewski 1997). The uterotrophic assay detects estrogenic activity by measuring the increase in uterine tissue mass in ovariectomized or immature laboratory rodents. It is considered by many to be the gold standard of estrogenicity assays because it targets a specific response at the physiologic level (Andersen et al. 1999; EDSTAC 1998; Korach and McLachlan 1995).

It might seem that determining estrogen equivalence would be relatively straightforward, requiring simply that the potency of a chemical be measured in one or more of the available estrogenicity assays and the potency compared with that of an endogenous estrogen such as estradiol-17 β . In practice, however, potency measurements among these assays can vary widely, making a determination of estrogen equivalence confusing and

uncertain (Table 2). Even assays that target the same biologic level of hormone action may produce disparate results. For example, Chen et al. (1997) showed relatively consistent results for transcription activation by isomers of DDT and its metabolites dichlorodiphenyl dichloroethane (DDD) and dichlorodiphenyl dichloroethylene (DDE) using two human ER- α (hER- α) reporter gene constructs. They reported relative potencies for *o,p'*-DDE of 7.7×10^{-4} to 1×10^{-3} and no activity for *p,p'*-DDE. In contrast, Tully et al. (2000) were unable to demonstrate transcription activation of an hER- α construct in human carcinoma cells by either DDT isomer or any of its metabolites. Balaguer et al. (1999) showed relative potencies for transcriptional activation of hER- α by DDE isomers in the range of 4×10^{-6} to 7×10^{-6} , while their relative potencies for ER- β were in the range of 4×10^{-6} to 4×10^{-5} . DDT metabolites and some hydroxylated PCBs have been shown to stimulate proliferative responses in estrogen-sensitive cells *in vitro* and *in vivo* (Table 2). Few *in vivo* studies using these chemicals have compared the responses to those produced by an endogenous estrogen, so the data do not allow an estimate of relative estrogenic potency.

Not only can potency measurements vary among estrogenicity assays, but hormone receptor specificity may also be unclear for some chemicals. Estrogenic and antiestrogenic effects have been reported for various chlorinated hydrocarbons (Safe 1995), some of which may be found in human milk (LaKind et al. 2001). A recent, prominent example is the ability of DDT isomers and metabolites that show activity in estrogenicity assays to also interact with the androgen receptor in human hepatoma cells transfected with a human androgen receptor-reporter gene construct (Maness et al. 1998). *o,p'*-DDE, the most potent estrogen agonist among the metabolites, antagonized androgen-stimulated transcription at concentrations similar to those shown to have estrogen agonist activity. Not only is it unclear how to characterize the relative estrogenic potency of *o,p'*-DDE (estimates range 2–3 orders of magnitude in various ER transcription activation assays), but it is also unclear whether this chemical is more potent as an estrogen or an antiandrogen. Thus, it is difficult to unambiguously categorize the hormonal activity or estimate the hormonal potency of *o,p'*-DDE based on the available data. Some PCB congeners have also recently been reported to be capable of interacting with both estrogen and androgen receptors *in vitro* at physiologically relevant concentrations (Bonefeld-Jorgensen et al. 2001). Whether effects mediated through androgen receptors or ERs manifest changes in endocrine function in intact organisms has not been determined.

Thus, for *o,p'*-DDE and many other chemicals such as PCBs, estimating estrogenic potency based on mechanistic information alone does not seem supportable at this time.

Discrepancies in potency estimates are also apparent in measurements of transcription activation by phytoestrogens. Like DDE, some phytoestrogens show greater affinity for ER- β than for ER- α ; for coumestrol and genistein these differences are 2-fold and 7-fold, respectively (Whitten and Patisaul 2001). Differential affinity for ER- α versus ER- β may have functional significance because the distribution of the two receptor isoforms varies among tissues and during development (Chang and Prins 1999; Gustafsson 2000). Furthermore, ER- α appears to be the most prevalent ER isoform in standard binding assays, suggesting that the estrogenic potency of selected phytoestrogens might be underestimated. Despite the greater affinity of genistein for ER- β , recombinant assay systems (e.g., yeast cells) transfected with one or the other ER isoform suggest that this phytoestrogen is more effective in activating ER- α -transfected cells than ER- β -transfected cells. In the former, genistein behaves as a full agonist, whereas in the latter it exhibits partial agonist activity (Whitten and Patisaul 2001).

In vitro proliferation assays reveal that phytoestrogens may either stimulate proliferation (estrogenic) or inhibit proliferation (antiestrogenic), a characteristic that is dependent on the assay system used, their concentration, and their relative potency. Some phytoestrogens (e.g., coumestrol and genistein) exhibit biphasic activity, being proliferative at low concentrations and inhibitory at higher concentrations. β -Zearalenol has been reported to exhibit triphasic activity, inhibiting estrogen activity at low and high concentrations and enhancing such activity at intermediate concentrations (Whitten and Patisaul 2001).

Potency estimates relative to estradiol exhibit marked discrepancies between assay systems and, in select cases, within assay systems. For example, ER-binding assays indicate that coumestrol has a potency relative to estradiol ranging from 11 to 185%, whereas in transactivation assays and proliferative assays (e.g., E-screen assays), the relative potency of this phytoestrogen is much less (less than 0.1%). As a matter of fact, as shown in Table 2, when binding assays, *in vitro* functional assays, and *in vivo* end points are compared, discrepancies are evident for other phytoestrogens (e.g., coumestrol) and mycoestrogens (α -zearalenone) as well as for genistein (Whitten and Patisaul 2001).

Whitten and Patisaul (2001) report that studies have been negative for proliferation of uterine and vaginal epithelium in rhesus macaques and rats fed a diet containing the phytoestrogen genistein. They also report that, in contrast, other studies have associated natural dietary exposure to phytoestrogens with

abnormalities in a number of estrogen-mediated processes in various livestock (e.g., cystic ovaries and estrus cycle irregularities). Phytoestrogen action in laboratory animals appears to depend on the hormonal milieu, and so opposite results might be obtained in pre- versus postpubertal animals. Of eight phytoestrogens tested, coumestrol, genistein, and daidzein appear to be the most potent to stimulate vaginal and uterine growth *in vivo* in sheep and mice. Relative to estradiol, the potency of daidzein and genistein is usually 0.1 to 0.2%, whereas that of coumestrol varies from less than 0.1% to as much as 20% (Whitten and Patisaul 2001).

Potency Variation Data and Implications for Risk Assessment

In the previous section, we discussed the various reasons that a mechanistic risk assessment approach is currently unsupportable for mixtures of estrogenic chemicals. However, it is important also to discuss the biologic basis of these reasons so that future research can be directed toward answering the questions critical to risk assessment. This section discusses in some detail the numerous reasons that biologic potency estimates vary for estrogenic chemicals.

The available assays for estrogenicity reflect a diverse array of molecular elements, biochemical pathways, and physiologic processes that mediate the action of endogenous estrogens in different tissues, organs, and organisms. At the molecular level, there are at least four distinct estrogenic mechanisms: classical ligand-dependent, ligand-independent, DNA binding-dependent, and cell-surface (nongenomic) signaling (Hall et al. 2001; Nadal et al. 2001). Assays that test different molecular mechanisms might be expected to exhibit dissimilar dose–response characteristics, and none of those could necessarily be expected to exhibit the same dose–response relationships as the integrated physiologic response observed in an intact organism. Furthermore, because different estrogenic pathways can mediate physiologic responses in different tissues and organs, it also seems unlikely that different physiologic responses would exhibit the same dose–response characteristics.

Each type of estrogenic assay, be it *in vivo* or *in vitro*, has distinct characteristics that may be an advantage or a detriment, depending on the particular objective of the study in question. An *in vivo* assay conducted in animals, such as the uterotrophic assay, assesses the net systemic biologic effects of bioactivation, detoxification, and pharmacokinetic processes as an integral part of the assay (Andersen et al. 1999; EDSTAC 1998; Zacharewski 1997). Although the uterotrophic assay may provide an indication of relatively short-term estrogenic effects (3–5 days' treatment), it may be unable to detect subtle

Table 2. Variability in potency estimates for xenoestrogens.

Publication/construct or assay chemical	Concentration (nM/L except as noted)	Quantitative response	Relative potency
Chen et al. 1997: hER reporter in yeast/Miller units β -galactosidase enzyme activity			
Estradiol-17 β (reference chemical)	1	0.4360	1.00E + 00
<i>o,p'</i> -DDT	50	0.3960	9.08E - 03
<i>o,p'</i> -DDT	1,000	0.6550	7.51E - 04
<i>o,p'</i> -DDE	1,000	0.6750	7.74E - 04
<i>o,p'</i> -DDD	1,000	0.5810	6.66E - 04
<i>p,p'</i> -DDT	1,000	0.4950	5.68E - 04
<i>p,p'</i> -DDE	1,000	0.0000	0.00E + 00
<i>p,p'</i> -DDA	1,000	0.0000	0.00E + 00
Chen et al. 1997: LexA-hER in CTY10-5d yeast/Miller units β -galactosidase			
Estradiol-17 β (reference chemical)	1	0.9800	1.00E + 00
<i>o,p'</i> -DDT	1,000	0.1100	1.12E - 04
<i>o,p'</i> -DDE	1,000	0.1010	1.03E - 04
<i>o,p'</i> -DDD	1,000	0.1050	1.07E - 04
<i>p,p'</i> -DDT	1,000	0.0977	9.97E - 05
<i>p,p'</i> -DDE	1,000	0.0000	0.00E + 00
<i>p,p'</i> -DDA	1,000	0.0000	0.00E + 00
Tully et al. 2000: pERET81CAT in HeLa cells/CAT protein immunoassay			
Estradiol-17 β (reference chemical)	0	0.0000	1.00E + 00
Estradiol-17 β (reference chemical)	10	0.2200	1.00E + 00
Estradiol-17 β (reference chemical)	100	0.4000	1.00E + 00
Estradiol-17 β (reference chemical)	1,000	1.0000	1.00E + 00
<i>p,p'</i> -DDT	1	0.0000	0.00E + 00
<i>p,p'</i> -DDT	10	0.0000	0.00E + 00
<i>p,p'</i> -DDT	100	0.0000	0.00E + 00
<i>p,p'</i> -DDT	1,000	0.0000	0.00E + 00
<i>p,p'</i> -DDT	10,000	0.0000	0.00E + 00
<i>p,p'</i> -DDD	1	0.0000	0.00E + 00
<i>p,p'</i> -DDD	10	0.0000	0.00E + 00
<i>p,p'</i> -DDD	100	0.0000	0.00E + 00
<i>p,p'</i> -DDD	1,000	0.0000	0.00E + 00
<i>p,p'</i> -DDD	10,000	0.0000	0.00E + 00
<i>p,p'</i> -DDE	1	0.0000	0.00E + 00
<i>p,p'</i> -DDE	10	0.0000	0.00E + 00
<i>p,p'</i> -DDE	100	0.0000	0.00E + 00
<i>p,p'</i> -DDE	1,000	0.0000	0.00E + 00
<i>p,p'</i> -DDE	10,000	0.0000	0.00E + 00
<i>p,p'</i> -DDT + <i>p,p'</i> -DDD	2	0.0000	0.00E + 00
<i>p,p'</i> -DDT + <i>p,p'</i> -DDD	20	0.0000	0.00E + 00
<i>p,p'</i> -DDT + <i>p,p'</i> -DDD	200	0.0000	0.00E + 00
<i>p,p'</i> -DDT + <i>p,p'</i> -DDD	2,000	0.0000	0.00E + 00
<i>p,p'</i> -DDT + <i>p,p'</i> -DDD	20,000	0.0000	0.00E + 00
<i>p,p'</i> -DDT + <i>p,p'</i> -DDE	2	0.0000	0.00E + 00
<i>p,p'</i> -DDT + <i>p,p'</i> -DDE	20	0.0000	0.00E + 00
<i>p,p'</i> -DDT + <i>p,p'</i> -DDE	200	0.0000	0.00E + 00
<i>p,p'</i> -DDT + <i>p,p'</i> -DDE	2,000	0.0000	0.00E + 00
<i>p,p'</i> -DDT + <i>p,p'</i> -DDE	20,000	0.0000	0.00E + 00
<i>p,p'</i> -DDD + <i>p,p'</i> -DDE	2	0.0000	0.00E + 00
<i>p,p'</i> -DDD + <i>p,p'</i> -DDE	20	0.0000	0.00E + 00
<i>p,p'</i> -DDD + <i>p,p'</i> -DDE	200	0.0000	0.00E + 00
<i>p,p'</i> -DDD + <i>p,p'</i> -DDE	2,000	0.0000	0.00E + 00
<i>p,p'</i> -DDD + <i>p,p'</i> -DDE	20,000	0.0000	0.00E + 00
Fielden et al. 1997: Specific binding, mouse uterine cytosol			
Estradiol-17 β (reference chemical)		IC ₅₀	1.00E + 00
PCB-104	1,700	IC ₅₀	9.09E - 03
OH-PCB-104	700	IC ₅₀	2.22E - 01
PCB-155	5,600	IC ₅₀	2.76E - 03
Fielden et al. 1997: Gal4-hER in MCF-7 cells/induction of luciferase			
Estradiol-17 β (reference chemical)	50	69	1.00E + 00
PCB-104	10,000	31.00	2.25E - 03
OH-PCB-104	1,000	15.00	1.09E - 02
OH-PCB-104	10,000	9.00	6.52E - 04
PCB-155	10,000	0.00	0.00E + 00
Balaguer et al. 1999: MELN (hER- α)/luciferase activity per milligram protein			
Estradiol-17 β (reference chemical)	0.1	1.00	1.00E + 00
<i>o,p'</i> -DDE	10,000	0.72	7.20E - 06
<i>p,p'</i> -DDE	10,000	0.40	4.00E - 06
2,3,7,8-TCDD	1,000	0.30	3.00E - 05

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Table 2. Continued.

Publication/construct or assay chemical	Concentration (nM/L except as noted)	Quantitative response	Relative potency
Balaguer et al. 1999: HELN α (hER- α)/luciferase activity per milligram protein			
Estradiol-17 β (reference chemical)	0.1	1.00	1.00E + 00
<i>o,p'</i> -DDE	10,000	0.70	7.00E - 06
<i>p,p'</i> -DDE	10,000	0.42	4.20E - 06
2,3,7,8-TCDD	1,000	-0.10	-1.00E - 05
Balaguer et al. 1999: HELN β (hER- β)/luciferase activity per milligram protein			
Estradiol-17 β (reference chemical)	1	1.00	1.00E + 00
<i>o,p'</i> -DDE	10,000	0.40	4.00E - 05
<i>p,p'</i> -DDE	10,000	0.36	3.60E - 05
2,3,7,8-TCDD	1,000	0.00	0.00E + 00
Garner et al. 1999: pERET81CAT in HeLa cells/CAT protein immunoassay			
Estradiol-17 β (reference chemical)	10	1.00	1.00E + 00
2,5-Dichloro-4'-biphenylol	10,000	0.20	2.03E - 04
2,4,6-Trichloro-4'-biphenyldiol	10,000	0.44	4.42E - 04
3,4-Biphenyldiol	50,000	0.42	8.32E - 05
2,5-Dichloro-3',4'-biphenyldiol	10,000	0.19	1.86E - 04
2,5-Dichloro-2',3'-biphenyldiol	10,000	0.06	5.62E - 05
2,4,6-Trichloro-3',4'-biphenyldiol	10,000	0.36	3.57E - 04
Gierthy et al. 1997: Induction of MCF-7 cells resulting in cellular aggregation or multilayered nodules of cells (foci)			
Estradiol-17 β (reference chemical)	1	1.00	1.00E + 00
2-Chlorobiphenyl	5,000	0.29	5.80E - 05
2-Chloro-4-biphenylol	5,000	0.21	4.20E - 05
4-Chlorobiphenyl	5,000	0.10	2.00E - 05
4-Chloro-4'-biphenylol	5,000	0.35	7.00E - 05
2,5-Dichlorobiphenyl	5,000	0.44	8.80E - 05
2,5-Dichloro-4-biphenylol	5,000	0.76	1.52E - 04
2,5-Dichloro-4-biphenylol	5,000	-0.13	-2.60E - 05
3,5-Dichlorobiphenyl	5,000	0.46	9.20E - 05
3,5-Dichloro-4'-biphenylol	5,000	0.44	8.80E - 05
3,5-Dichloro-4-biphenylol	5,000	-0.13	-2.60E - 05
2,4,6-Trichlorobiphenyl	5,000	0.48	9.60E - 05
2,4,6-Trichloro-4'-biphenylol	5,000	0.98	1.96E - 04
3,4,5-Trichlorobiphenyl	5,000	0.2000	4.00E - 05
3,4,5-Trichlorobiphenyl	5,000	-0.4000	-8.00E - 05
3,4,5-Trichloro-4-biphenylol	5,000	1.0000	2.00E - 04
Korach et al. 1988: Specific binding, murine uterine cytosol			
Estradiol-17 β (reference chemical)	N/D	N/D	1.00E + 00
4-Hydroxy-2',3',4',5'-tetrachlorobiphenyl	N/D	N/D	2.38E - 02
4-Hydroxy-2',4',6'-trichlorobiphenyl	N/D	N/D	1.05E - 02
4,4'-Dihydroxy-2'-chlorobiphenyl	N/D	N/D	1.11E - 02
4-Hydroxy-2',6'-dichlorobiphenyl	N/D	N/D	2.58E - 03
4-Hydroxy-2',5'-dichlorobiphenyl	N/D	N/D	1.98E - 03
4-Hydroxy-3,5,4'-trichlorobiphenyl	N/D	N/D	1.00E - 03
4,4'-Dihydroxy-3,5,3',5'-tetrachlorobiphenyl	N/D	N/D	7.39E - 04
4-Hydroxy-2-chlorobiphenyl	N/D	N/D	4.00E - 04
4-Hydroxy-4'-chlorobiphenyl	N/D	N/D	2.56E - 04
4,4'-Dihydroxy-2',3',5',6'-tetrachlorobiphenyl	N/D	N/D	2.00E - 04
4,4'-Dihydroxybiphenyl	N/D	N/D	< 2.00E - 04
4-Hydroxybiphenyl	N/D	N/D	< 2.00E - 04
Bonefeld-Jorgensen et al. 2001: pERE-tk-CAT in MCF-7 cells/CAT protein immunoassay			
Estradiol-17 β (reference chemical)	10	1.000000	1.00E + 00
PCB-138	9,000	-0.610000	-6.78E - 04
PCB-153	9,000	-0.500000	-5.56E - 04
PCB-180	9,000	-0.790000	-8.78E - 04
PCB-138 + E ₂	9,000 + 10	-0.360000	-4.00E - 04
PCB-153 + E ₂	9,000 + 10	-0.410000	-4.56E - 04
PCB-180 + E ₂	9,000 + 10	-0.750000	-8.33E - 04
PCB-138 + PCB-153 + PCB-180	3,000 + 3,000 + 3,000	-0.420000	-4.67E - 04
PCB-138 + PCB-153 + PCB-180 = E ₂	3,000 + 3,000 + 3,000 + 10	-0.270000	-3.00E - 04
Smeets et al. 1999: Vitellogenin induction in Carp hepatocyte cells			
Estradiol-17 β (reference chemical)	2	LOEC	1.00E + 00
DES	6	LOEC	5.00E - 01
Methoxychlor	5,000	LOEC	1.00E - 03
<i>o,p'</i> -DDT	25,000	LOEC	2.00E - 04
Chlordecone	20,000	LOEC	1.00E - 04
Bisphenol A	50,000	LOEC	1.00E - 04
4- <i>t</i> -Pentylphenol	50,000	LOEC	1.00E - 04
<i>o,p'</i> -DDE	ND	LOEC	ND
Toxaphene	ND	LOEC	ND
β -Endosulfan	ND	LOEC	ND
Dieldrin	ND	LOEC	ND

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Table 2. Continued.

Publication/construct or assay chemical	Concentration (nM/L except as noted)	Quantitative response	Relative potency
Metcalf et al. 2001: Yeast estrogen screen assay—effective concentration inducing 20% of maximum response observed with E ₂			
Estradiol-17β (reference chemical)	0.022000	EC ₂₀ (ng/mL)	1.00E + 00
Ethinylestradiol-17α	0.059	EC ₂₀ (ng/mL)	3.80E - 01
Estrone	0.166	EC ₂₀ (ng/mL)	1.40E - 01
Estriol	0.627	EC ₂₀ (ng/mL)	3.70E - 02
Nonylphenol	246.7	EC ₂₀ (ng/mL)	8.90E - 05
Nonylphenol monoethoxylate	10,985.4	EC ₂₀ (ng/mL)	2.00E - 06
Nonylphenol monoethoxylate/nonylphenol diethoxylate	9,627.6	EC ₂₀ (ng/mL)	2.30E - 06
Nonylphenol monoethoxycarboxylate	0.0	EC ₂₀ (ng/mL)	0.00E + 00
Nonylphenol monoethoxycarboxylate/nonylphenol diethoxycarboxylate	0.0	EC ₂₀ (ng/mL)	0.00E + 00
Bisphenol A	597.3	EC ₂₀ (ng/mL)	3.70E - 05
Diethylhexyl phthalate	0.0	EC ₂₀ (ng/mL)	0.00E + 00
Madigou et al. 2001: Rainbow trout hepatocyte culture/induction of vitellogenin mRNA			
Estradiol-17β (reference chemical)	1,000	100	1.00E + 00
4- <i>n</i> -Nonylphenol	10,000	22	2.20E - 02
4- <i>n</i> -Nonylphenol diethoxylate	10,000	0	0.00E + 00
4- <i>n</i> -Nonylphenol diethoxylate	100,000	0	0.00E + 00
Nonylphenol glucuronide	1,000	0	0.00E + 00
Nonylphenol glucuronide	10,000	0	0.00E + 00
3-(4-Hydroxyphenol)-propionic acid	10,000	0	0.00E + 00
3-(4-Hydroxyphenol)-propionic acid	100,000	0	0.00E + 00
4-Hydroxybenzoic acid	10,000	0	0.00E + 00
4-Hydroxybenzoic acid	100,000	0	0.00E + 00
Madigou et al. 2001: 2ERE-CYC1-lacZ in yeast/Miller units β-galactosidase			
Estradiol-17β (reference chemical)	100	100	1.00E + 00
4- <i>n</i> -Nonylphenol	10,000	92	9.20E - 03
4- <i>n</i> -Nonylphenol diethoxylate	100,000	0	0.00E + 00
Nonylphenol glucuronide	100,000	0	0.00E + 00
3-(4-Hydroxyphenol)-propionic acid	100,000	0	0.00E + 00
4-Hydroxybenzoic acid	100,000	0	0.00E + 00
Andersen et al. 1999: Recombinant hER in MCF-7 cells/direct competitive binding			
Estradiol-17β (reference chemical)	1	IC ₅₀	1.00E + 00
Ethinyl estradiol-17α (standard)	0.67	IC ₅₀	2.20E + 00
DES	0.25	IC ₅₀	5.80E + 00
Tamoxifen	2.6	IC ₅₀	6.00E - 01
ICI 182.780	3.6	IC ₅₀	4.00E - 01
Testosterone	> 200,000	IC ₅₀	
Bisphenol A	11,000	IC ₅₀	1.30E - 04
Bisphenol A dimethacrylate	> 0.00000002	IC ₅₀	
4- <i>n</i> -OP	4,000	IC ₅₀	3.60E - 04
4- <i>n</i> -NP	4,300	IC ₅₀	3.40E - 04
NP12EO	57,000	IC ₅₀	2.50E - 05
BBP	12,000	IC ₅₀	1.20E - 04
DBP	> 200,000	IC ₅₀	
Methoxychlor	> 200,000	IC ₅₀	
<i>o,p</i> -DDT	5	IC ₅₀	2.90E - 03
<i>p,p'</i> -DDE	16,000	IC ₅₀	9.10E - 05
Endosulfan	13,000	IC ₅₀	1.20E - 04
Chlormequat chloride	56,000	IC ₅₀	2.60E - 05
Colchicine	> 200,000	IC ₅₀	
Andersen et al. 1999: Rabbit uterine tissue/ <i>In vitro</i> ER binding assay			
Estradiol-17β (reference chemical)	0.02	IC ₅₀	1.00E + 00
Ethinyl estradiol-17α (standard)	0.002	IC ₅₀	1.00E + 01
DES	0.000007	IC ₅₀	2.86E + 03
Tamoxifen	120	IC ₅₀	1.70E - 04
ICI 182.780	0.004	IC ₅₀	5.00E + 00
Testosterone	> 10,000	IC ₅₀	
Bisphenol A	1,600	IC ₅₀	1.30E - 05
Bisphenol A dimethacrylate	4,300	IC ₅₀	4.70E - 06
4- <i>n</i> -OP	> 10,000	IC ₅₀	
4- <i>n</i> -NP	1,800	IC ₅₀	1.10E - 05
NP12EO	> 10,000	IC ₅₀	
BBP	> 10,000	IC ₅₀	
DBP	> 10,000	IC ₅₀	
Methoxychlor	6,500	IC ₅₀	3.10E - 06
<i>o,p</i> -DDT	3,400	IC ₅₀	5.90E - 06
<i>p,p'</i> -DDE	> 10,000	IC ₅₀	
Endosulfan	> 10,000	IC ₅₀	
Chlormequat chloride	> 10,000	IC ₅₀	
Colchicine	> 10,000	IC ₅₀	

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Table 2. Continued.

Publication/construct or assay chemical	Concentration (nM/L except as noted)	Quantitative response	Relative potency
Le Guevel and Pakdel 2001: Recombinant yeast expressing hER/ β -galactosidase induction			
Ethynyl estradiol-17 α (reference chemical)	1	EC ₅₀	1.00E + 00
DES	2.9	EC ₅₀	2.10E - 01
Estradiol-17 β	0.74	EC ₅₀	8.30E - 01
Estradiol-17 α	5.2	EC ₅₀	1.20E - 01
Estrone	2.1	EC ₅₀	2.90E - 01
Zearalenone	130	EC ₅₀	5.00E - 03
Zeralanone	110	EC ₅₀	6.00E - 03
α -Zearalenol	30	EC ₅₀	2.20E - 02
β -Zearalenol	280	EC ₅₀	0.00E + 00
α -Zearalanol	4,000	EC ₅₀	1.40E - 02
β -Zearalanol	160	EC ₅₀	4.00E - 03
Le Guevel and Pakdel 2001: Rainbow trout ER in recombinant yeast/ β -galactosidase units			
Ethynyl estradiol-17 α (reference chemical)	3.6	EC ₅₀	1.00E + 00
DES	4.3	EC ₅₀	8.20E - 01
Estradiol-17 β	5.2	EC ₅₀	6.80E - 01
Estradiol-17 α	140	EC ₅₀	3.00E - 02
Estrone	22	EC ₅₀	1.60E - 01
Zearalenone	62	EC ₅₀	6.00E - 02
Zeralanone	32	EC ₅₀	1.10E - 01
α -Zearalenol	12	EC ₅₀	2.90E - 01
β -Zearalenol	> 50,000	EC ₅₀	> 1.00E - 04
α -Zearalanol	30	EC ₅₀	1.10E - 01
β -Zearalanol	110	EC ₅₀	3.00E - 02
Behnisch et al. 2001: E-screen assay with calf MCF-7 cells			
Estradiol-17 β (standard)	0.0062	EC ₅₀	1.00E + 00
Ethynyl estradiol-17 α (reference chemical)	0.0021	EC ₅₀	3.00E + 00
Bisphenol A	110	EC ₅₀	5.60E - 04
Butyl benzylphthalate	490	EC ₅₀	1.30E - 05
Di- <i>n</i> -butylphthalate	1,700	EC ₅₀	3.60E - 06
4-Octylphenol	320	EC ₅₀	1.90E - 05
Nikov et al. 2000: Phytoestrogen binding to hER- α			
Estradiol-17 β (reference chemical)	13 \pm 0.7	IC ₅₀	1.00E + 00
Genistein	825 \pm 2	IC ₅₀	1.60E - 02
Coumestrol	109 \pm 1	IC ₅₀	1.20E - 01
Zearalenone	59 \pm 0.8	IC ₅₀	2.20E - 01
Daidzein	7 \pm 1	IC ₅₀	2.00E - 03
Glyceolin	6 \pm 0.6	IC ₅₀	2.20E - 03
Testosterone	35 \pm 0.5	IC ₅₀	4.00E - 04
Nikov et al. 2000: Phytoestrogen binding to hER- β			
Estradiol-17 β (reference chemical)	12 \pm 0.5	IC ₅₀	1.00E + 00
Genistein	12 \pm 0.7	IC ₅₀	1.00E + 00
Coumestrol	35 \pm 0.7	IC ₅₀	3.40E - 01
Zearalenone	16 \pm 0.5	IC ₅₀	7.50E - 01
Daidzein	670 \pm 1	IC ₅₀	1.80E - 02
Glyceolin	16 \pm 1.4	IC ₅₀	8.00E - 04
Testosterone	20 \pm 1	IC ₅₀	6.00E - 04
Harper et al. 1994: Progesterone receptor induction in MCF-7 human breast cancer cells			
Estradiol-17 β (reference chemical)	1	283	1.00E + 00
TCDD	1	84	2.97E - 01
Eroschenko et al. 2000: Reproductive tract weight in ovariectomized adult mice; note concentrations in nanograms.			
Estradiol-17 β (reference chemical)	25	1.6	1.00E + 00
Methoxychlor	125,000,000	1.1	1.38E - 07
Danzo 1997: Percent binding to rabbit uterine cytosol ERs using the charcoal assay procedure			
Estradiol-17 β (reference chemical)			1.00E + 00
5 α -Dihydrotestosterone			8.50E - 01
Hexachlorocyclohexane			1.15E + 00
Hexachlorocyclohexane			1.19E + 00
Methoxychlor			8.80E - 01
<i>p</i> , <i>p'</i> -DDE			9.00E - 01
DDE			9.20E - 01
<i>o</i> , <i>p</i> -DDT			4.00E - 01
Dieldrin			9.70E - 01
Atrazine			9.80E - 01
Pentachlorophenol			8.20E - 01
Nonylphenol			2.50E - 01

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Table 2. Continued.

Publication/construct or assay chemical	Concentration (nM/L except as noted)	Quantitative response	Relative potency
Matthews et al. 2000: Binding to human α -GST ERs			
Estradiol-17 β (reference chemical)	0.29	IC ₅₀	1.00E + 00
4-Hydroxytamoxifen	1.90	IC ₅₀	1.55E + 00
Ethynyl estradiol	2.30	IC ₅₀	1.27E + 00
DES	3.20	IC ₅₀	9.10E - 01
α -Zearalanol	6.10	IC ₅₀	4.80E - 01
Estrone	6.50	IC ₅₀	4.50E - 01
ICI 164, 384	7.00	IC ₅₀	4.20E - 01
Estriol	10.00	IC ₅₀	2.80E - 01
β -Zearalanol	23.00	IC ₅₀	1.30E - 01
Tamoxifen	28.00	IC ₅₀	1.10E - 01
Estradiol benzoate	-9.00	IC ₅₀	1.00E - 01
Zearalenone	31.00	IC ₅₀	9.30E - 02
HPTE	250.00	IC ₅₀	1.20E - 02
Coumestrol	360.00	IC ₅₀	8.10E - 03
Genistein	630.00	IC ₅₀	4.60E - 03
4- <i>t</i> -Octylphenol	2,400.00	IC ₅₀	1.20E - 03
Dihydrotestosterone	5,900.00	IC ₅₀	4.90E - 04
Bisphenol A	36,000.00	IC ₅₀	8.00E - 05
Kepone	42,000.00	IC ₅₀	6.90E - 05
Matthews et al. 2000: Binding to mouse α -GST ERs			
Estradiol-17 β (reference chemical)	2.70	IC ₅₀	1.00E + 00
4-Hydroxytamoxifen	1.20	IC ₅₀	2.12E + 00
Ethynyl estradiol	2.20	IC ₅₀	1.18E + 00
DES	3.20	IC ₅₀	8.40E - 01
α -Zearalanol	5.10	IC ₅₀	5.30E - 01
Estrone	9.50	IC ₅₀	2.80E - 01
ICI 164, 384	5.90	IC ₅₀	4.50E - 01
Estriol	21.00	IC ₅₀	1.30E - 01
β -Zearalanol	24.00	IC ₅₀	1.10E - 01
Tamoxifen	26.00	IC ₅₀	1.00E - 01
Estradiol benzoate	23.00	IC ₅₀	1.20E - 01
Zearalenone	23.00	IC ₅₀	1.20E - 01
HPTE	220.00	IC ₅₀	1.20E - 02
Coumestrol	800.00	IC ₅₀	3.30E - 03
Genistein	810.00	IC ₅₀	3.30E - 03
4- <i>t</i> -Octylphenol	1,600.00	IC ₅₀	1.70E - 03
Dihydrotestosterone	6,600.00	IC ₅₀	4.00E - 04
Bisphenol A	31,000.00	IC ₅₀	8.60E - 05
Kepone	64,000.00	IC ₅₀	3.50E - 05
<i>o,p</i> -DDT	36,000.00	IC ₅₀	7.30E - 05
Matthews et al. 2000: Binding to chicken α -GST ERs			
Estradiol-17 β (reference chemical)	3.20	IC ₅₀	1.00E + 00
4-Hydroxytamoxifen	1.90	IC ₅₀	1.68E + 00
Ethynyl estradiol	1.90	IC ₅₀	1.71E + 00
DES	2.50	IC ₅₀	1.30E + 00
α -Zearalanol	4.60	IC ₅₀	7.00E - 01
Estrone	6.40	IC ₅₀	5.00E - 01
ICI 164, 384	5.20	IC ₅₀	6.20E - 01
Estriol	29.00	IC ₅₀	1.10E - 01
β -Zearalanol	14.00	IC ₅₀	2.30E - 01
Tamoxifen	21.00	IC ₅₀	1.60E - 01
Estradiol benzoate	22.00	IC ₅₀	1.50E - 01
Zearalenone	9.90	IC ₅₀	3.30E - 01
HPTE	68.00	IC ₅₀	4.80E - 02
Coumestrol	460.00	IC ₅₀	7.00E - 03
Genistein	410.00	IC ₅₀	7.80E - 03
4- <i>t</i> -Octylphenol	560.00	IC ₅₀	5.70E - 03
Dihydrotestosterone	38,000.00	IC ₅₀	8.50E - 05
Bisphenol A	7,300.00	IC ₅₀	4.40E - 04
Kepone	30,000.00	IC ₅₀	1.10E - 03
<i>o,p</i> -DDT	37,000.00	IC ₅₀	8.60E - 04
Quercetin	82,000.00	IC ₅₀	3.90E - 05
Naringenin	39,000.00	IC ₅₀	8.20E - 05
Matthews et al. 2000: Binding to green anole α -GST ERs			
Estradiol-17 β (reference chemical)	3.10	IC ₅₀	1.00E + 00
4-Hydroxytamoxifen	1.30	IC ₅₀	2.43E + 00
Ethynyl estradiol	2.20	IC ₅₀	1.39E + 00
Diethylstilbestrol	2.90	IC ₅₀	1.07E + 00
α -Zearalanol	8.60	IC ₅₀	3.60E - 01
Estrone	5.10	IC ₅₀	6.00E - 01

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Table 2. Continued.

Publication/construct or assay chemical	Concentration (nM/L except as noted)	Quantitative response	Relative potency
ICI 164, 384	11.00	IC ₅₀	2.80E – 01
Estriol	10.00	IC ₅₀	3.00E – 01
β-Zearalanol	73.00	IC ₅₀	4.20E – 02
Tamoxifen	30.00	IC ₅₀	1.00E – 01
Estradiol benzoate	24.00	IC ₅₀	1.30E – 01
Zearalenone	27.00	IC ₅₀	1.20E – 01
HPTE	64.00	IC ₅₀	4.80E – 02
Coumestrol	100.00	IC ₅₀	3.10E – 02
Genistein	240.00	IC ₅₀	1.30E – 02
4- <i>t</i> -Octylphenol	3,900.00	IC ₅₀	7.90E – 04
Dihydrotestosterone	820.00	IC ₅₀	3.80E – 03
Bisphenol A	2,400.00	IC ₅₀	1.30E – 03
Kepone	27,000.00	IC ₅₀	1.10E – 04
Quercitin	19,000.00	IC ₅₀	1.60E – 04
Naringenin	4,700.00	IC ₅₀	6.50E – 04
Matthews et al. 2000: Binding to rainbow trout α-GST ERs			
Estradiol-17β (reference chemical)	3.30	IC ₅₀	1.00E + 00
4-Hydroxytamoxifen	1.20	IC ₅₀	2.72E + 00
Ethynyl estradiol	3.10	IC ₅₀	1.08E + 00
DES	2.00	IC ₅₀	1.65E + 00
α-Zearalanol	1.30	IC ₅₀	2.67E + 00
Estrone	24.00	IC ₅₀	1.40E – 01
ICI 164, 384	1.00	IC ₅₀	3.27E + 00
Estriol	90.00	IC ₅₀	3.70E – 02
β-Zearalanol	3.70	IC ₅₀	9.10E – 01
Tamoxifen	13.00	IC ₅₀	2.50E – 01
Estradiol benzoate	3.70	IC ₅₀	9.00E – 02
Zearalenone	4.10	IC ₅₀	8.20E – 01
HPTE	24.00	IC ₅₀	1.40E – 01
Coumestrol	1,400.00	IC ₅₀	2.40E – 03
Genistein	750.00	IC ₅₀	4.40E – 03
4- <i>t</i> -Octylphenol	1.10	IC ₅₀	3.20E – 02
Dihydrotestosterone	10,000.00	IC ₅₀	3.40E – 04
Bisphenol A	1,600.00	IC ₅₀	2.10E – 03
Kepone	6,200.00	IC ₅₀	5.40E – 04
Naringenin	8,700.00	IC ₅₀	3.90E – 04
DHEA	12,000.00	IC ₅₀	2.80E – 04
Quercitin	8,000.00	IC ₅₀	4.20E – 04
<i>o,p'</i> -DDT	780.00	IC ₅₀	4.30E – 03
<i>o,p'</i> -DDE	3,200.00	IC ₅₀	1.10E – 03
<i>p,p'</i> -DDE	8,000.00	IC ₅₀	4.20E – 04
Dibutylbenzylphthalate	1,700.00	IC ₅₀	2.00E – 03
α-Endosulfan	28,000.00	IC ₅₀	1.20E – 04
Methoxychlor	3,500.00	IC ₅₀	9.50E – 03
Diel et al. 2000: Uterotrophic assay in ovariectomized 14-day-old DA/Han rats			
Ethynyl estradiol-17α (reference chemical)	0.10	1,300	1.00E + 00
<i>o,p'</i> -DDT	10	42	3.23E – 04
<i>o,p'</i> -DDT	100	446	3.43E – 04
<i>o,p'</i> -DDT	500	665	1.02E – 04
Lemini et al. 1997: Uterotrophic activity in immature CD1 mice measured as uterine weight (mg)			
Estradiol-17β (reference chemical)	1 µg/100 g	64 ± 4.4	1.00E + 00
<i>p</i> -Hydroxybenzoic acid (PHBA)	500 µg/100 g	63 ± 4.5	1.10E – 03
Lemini et al. 1997: Uterotrophic activity in ovariectomized CD1 mice measured as uterine weight (mg)			
Estradiol-17β (reference chemical)	1 µg/100 g	137 ± 11	1.00E + 00
<i>p</i> -Hydroxybenzoic acid (PHBA)	500 µg/100 g	92 ± 6.5	1.80E – 03
Katsuda et al. 2000: Uterotrophic assay in adult Crj:Donryu rats measured as uterine weight (g)—2-day treatment			
Estradiol-17β (reference chemical)	0.005	0.618	1.00E + 00
<i>p-tert</i> -Octylphenol	6.25	0.150	1.94E – 04
<i>p-tert</i> -Octylphenol	12.5	0.147	9.51E – 05
<i>p-tert</i> -Octylphenol	25	0.175	5.66E – 05
<i>p-tert</i> -Octylphenol	50	0.202	3.27E – 05
<i>p-tert</i> -Octylphenol	100	0.285	2.31E – 05
<i>p-tert</i> -Octylphenol	200	0.381	1.54E – 05

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Table 2. Continued.

Publication/construct or assay chemical	Concentration (nM/L except as noted)	Quantitative response	Relative potency
Katsuda et al. 2000: Uterotrophic assay in adult Crj:Donryu rats measured as uterine weight (g)—14 day treatment			
Estradiol-17 β (reference chemical)	0.005	0.148	1.00E + 00
<i>p</i> -tert-Octylphenol	6.25	0.132	7.14E – 04
<i>p</i> -tert-Octylphenol	12.5	0.142	3.84E – 04
<i>p</i> -tert-Octylphenol	25	0.233	3.15E – 04
<i>p</i> -tert-Octylphenol	50	0.305	2.06E – 04
<i>p</i> -tert-Octylphenol	100	0.422	1.43E – 04
Santell et al. 1997: Uterotrophic assay in immature or adult ovariectomized Sprague-Dawley rats measured as uterine weight (mg)			
Estradiol-17 β (reference chemical)	0.5 μ g/kg	122.1	1.00E + 00
Estradiol-17 β (standard)	1.0 μ g/day	194.8	7.98E – 01
Estradiol-17 β (standard)	1.5 μ g/day	255	6.96E – 01
Genistein	150 μ g/day	92.4	2.52E – 03
Genistein	375 μ g/day	135.6	1.48E – 03
Genistein	750 μ g/day	189.3	1.03E – 03
Petroff et al. 2000: Uterotrophic assay in immature Sprague-Dawley rats measured as uterine weight (mg)			
Estradiol cypionate (reference chemical)	2	41.9	1.00E + 00
TCDD	0.010	38.1	1.82E + 02
Cummings and Laws 2000: Percent implanting in female Holtzman rats			
Estrone (reference chemical)	0.001 mg/kg	100	1.00E + 00
Methoxychlor	6.25 mg/kg	10	1.60E – 05
Methoxychlor	12.5 mg/kg	8	6.40E – 06
Methoxychlor	25 mg/kg	37	1.48E – 05
Methoxychlor	50 mg/kg	68	1.36E – 04
Methoxychlor	100 mg/kg	57	5.70E – 06
Methoxychlor	200 mg/kg	70	3.50E – 06
Methoxychlor	300 mg/kg	100	3.33E – 06
Bisphenol A	25 mg/kg	30	1.20E – 05
Bisphenol A	50 mg/kg	50	1.00E – 05
Bisphenol A	100 mg/kg	78	7.80E – 06
Bisphenol A	200 mg/kg	100	5.00E – 06
4-tert-Octylphenol	200 mg/kg	25	1.25E – 06
4-tert-Octylphenol	300 mg/kg	22	7.33E – 07
4-tert-Octylphenol	400 mg/kg	75	1.88E – 06
Jansen et al. 1993: Uterotrophic assay in immature Sprague-Dawley rats measured as uterine weight (mg)			
Estradiol-17 β (reference chemical)	1 μ g/day	60	1.00E + 00
PCB-77	160 μ g/day	25	2.60E – 03
PCB-52	640 μ g/day	32	8.33E – 04
OH-PCB	250 μ g/day	28	1.87E – 03
Aroclor 1242	80 μ g/day	31	6.46E – 03
Aroclor 1242	320 μ g/day	34	1.77E – 03
Fielden et al. 1997: Uterotrophic activity in ovariectomized CD1 mice measured as uterine weight (g)			
Ethynyl estradiol-17 α (reference chemical)	0.10	1.1	2.39E + 00
Ethynyl estradiol-17 α (reference chemical)	1.0	4.6	1.00E + 00
PCB-104	1.7	1.1	5.88E – 02
PCB-104	16	1.3	7.39E – 03
PCB-104	202	1.8	8.10E – 04
Carlson and Williams 2001: Plasma vitellogenin induction in rainbow trout			
Estradiol-17 β (reference chemical)			1.00E + 00
Estrone			1.00E + 00
OH-PCB-30			1.00E – 01
OH-PCB-61			1.00E – 03
PCB-30			0.00E + 00
PCB-61			0.00E + 00
PCB-75			0.00E + 00
PCB-114			0.00E + 00

Abbreviations: CAT, choline acetyltransferase; GST, glutathione *S*-transferase; h, human; LOEC, lowest observable effect concentration; ND, no data; VTG, vitellogenin; YES, yeast estrogen screen. Data from tables or visually extracted from graphs in selected publications were used to calculate relative estrogenic potencies according to the following formula: $[R_T/CT \times C_{STD}/R_{STD}]$, where R denotes the measured response, and C denotes the concentration of either the estrogenic standard (STD) or test chemical (T). Negative values indicate anti-estrogenic potencies, i.e., the ability of a chemical to antagonize the response to the estrogenic standard. For the mixtures studied by Bonefield-Jorgensen et al. (2001), the mathematical sum of the component concentrations was assumed for CT, including the estrogenic standard, i.e., 9,000 nM PCB + 10 nM E₂ was assumed to be 9,010 nM.

estrogenic effects that may occur after long-term, low-level exposures. A positive response in the uterotrophic assay requires higher levels of estradiol than *in vitro* receptor binding and transactivational assays (Zacharewski 1997), an aspect that has caused some to speculate that the uterotrophic assay may be unable to detect weakly estrogenic substances. Given this speculation, some have argued that potency estimates should be based on more sensitive *in vitro* assays to ensure that the potency of weak estrogens is incorporated into potency estimates for mixtures of estrogenic compounds (EDSTAC 1998; Payne et al. 2001). However, it seems that potency estimates obtained via animal assays would actually be a more appropriate prediction of what might occur under real-life circumstances than *in vitro* assays. In whole-animal assays, pharmacokinetic processes can influence the pharmacodynamic responses of integrated physiologic systems.

In vitro assays, on the other hand, are unable to fully assess a number of processes that determine the *in vivo* activity of endogenous hormones and exogenous chemicals, including metabolism, pharmacokinetics, and interaction with other hormones and hormonal systems (Andersen et al. 1999; Degen and Bolt 2000; Zacharewski 1997). Therefore, potency comparisons based on *in vitro* or short-term *in vivo* assays may not predict long-term effects in intact organisms. For this reason, such assays are typically regarded as useful screens (Andersen et al. 1999; Degen and Bolt 2000; EDSTAC 1998; Shelby et al. 1996) and it would seem that potency data derived from such studies could not be used in comparative risk assessments without detailed knowledge of the physiologic, biochemical, and pharmacokinetic properties of the chemical in the target organism.

The potential for systemic metabolic transformation to alter the activity of substances *in vivo* is of particular concern for estrogenic screening assays. Specifically, there has been concern that receptor binding, cell proliferation, and yeast-based transcription assays might produce false-negative or false-positive results for the prediction of estrogenic activity *in vivo* because of the absence of metabolic transformation of the chemicals in question by peripheral tissues. Such metabolic transformation may not only inactivate chemicals with hormonal activity, but can also metabolize a number of chemicals to active metabolites (Charles et al. 2000; Connor et al. 1997; Elsby et al. 2000, 2001a, 2001b; Ferruck et al. 2001; Garner et al. 1999; Nakagawa and Suzuki 2002; Sugihara et al. 2000). Active metabolites may have mechanisms of action and potencies different from the parent compound. *In vitro* assays that differ in metabolic capability can produce different relative potency measurements, depending on the chemical (Le Guevel

and Pakdel 2001). For example, a cell proliferation assay in Ishikawa cells and transcription activation assays in yeast assays produced EC₅₀ concentrations (concentrations that elicited 50% of the maximum effect) for estradiol-17 β that were within an order of magnitude, depending on whether the recombinant yeast assay utilized hER or rainbow trout ER. In those same assays, however, EC₅₀ concentrations differed by 3–4 orders of magnitude for the mycotoxin zearalenone, for individual metabolites of zearalenone, for various synthetic estrogens, and for diethylstilbestrol (DES). Although the absolute potency differences between chemicals were not identical in the yeast assay, incorporating recombinant ERs yields a much greater potency difference between estradiol-17 β and some of the synthetic estrogens than was observed in the Ishikawa cell proliferation assay (Le Guevel and Pakdel 2001).

Pharmacokinetic differences can also be sufficient to alter relative estrogenic potency at the physiologic level relative to the molecular level. Daidzein is a prime example of how inefficient gastrointestinal absorption and rapid urinary elimination alter estrogenic potency in rats (Bayer et al. 2001) relative to ER affinity or functional potency *in vitro* (Hopert et al. 1998).

In addition to differences in absorption and elimination kinetics, differential binding to serum proteins can create large differences in the cellular uptake and cellular response to different estrogens. Endogenous sex steroid hormones are bound extensively to plasma proteins, the most significant of which appears to be sex hormone binding globulin (SHBG). SHBG is a plasma glycoprotein that binds a number of circulating steroid hormones with high affinity, including testosterone, dihydrotestosterone, and estradiol. Because sex hormones bound to SHBG are restricted from crossing cell membranes to activate hormone receptors in target cells, SHBG is generally thought to regulate hormone action passively by controlling free plasma concentrations (reviewed in Westphal 1986). Because SHBG controls the delivery of the endogenous hormone to a target cell, it was suggested that the *in vivo* potency of endogenous hormones would be less than predicted by *in vitro* assays because of their sequestration by SHBG in plasma. Nagel et al. (1997) hypothesized that if xenoestrogens have a lower affinity for SHBG and access of estrogenic chemicals to the cell were less restricted, the potency of weak estrogens *in vivo* might be underestimated in standard *in vitro* assays. A number of phytoestrogens and industrial chemicals have been shown to bind SHBG with affinities far less than those of endogenous steroids (Hodgert Jury et al. 2000). Hodgert Jury et al. (2000) suggest that low-affinity binding may

be physiologically significant when SHBG levels are low, as occurs in prepubertal children, or are artificially reduced, as occurs with oral contraceptive use.

Nagel et al. (1998) examined the effect of plasma binding of a limited number of xenoestrogens by comparing their uptake by MCF-7 human breast cancer cells in the presence and absence of 100% adult male human serum. These studies revealed that plasma binding (and hence uptake) of such chemicals is highly variable and does not appear to be predictable by the chemical nature or type of substance involved. For example, the effective free fraction (or fraction unrestricted by plasma protein) varied by as much as 30-fold among the phytoestrogens and more than 60-fold among the synthetic estrogens. The effective free fraction of the SERM (selective ER modulator) raloxifene was 10-fold that of another SERM, tamoxifen. The effective free fraction of the alkylphenol nonylphenol was estimated to be 4-fold that of octylphenol. The influence of plasma binding also varied greatly among classes of xenoestrogens (Nagel et al. 1998). Similarly, a significant variation in the degree of SHBG binding of xenoestrogens is suggested by the findings of Hodgert Jury et al. (2000) using a different methodology (ammonium sulfate precipitation method).

Although the ER is quite promiscuous, having the ability to bind a chemically diverse array of substances (Blair et al. 2000), binding *per se* does not explain the qualitative nature of a biologic response resulting from such a ligand-ER interaction. Such a response could be estrogenic (or agonistic), mimicking the action of estrogen, or can be antiestrogenic (or antagonistic). The quality (or direction) of such a response appears to be a function of the particular ligand and target tissue involved. This dependence on substance and target tissue is exemplified dramatically by a comparison of the response profile of estradiol with those of two therapeutic agents, tamoxifen and raloxifene, on four specific end points: cell proliferation in the breast, cell proliferation in the uterus, blood levels of LDL cholesterol, and bone density (or prevention of osteoporosis). Estradiol stimulates breast and uterine cell proliferation, lowers blood LDL cholesterol, and maintains bone density. Tamoxifen is antiestrogenic in breast, but mimics the action of estradiol in uterus, blood LDL cholesterol, and bone. Raloxifene, on the other hand, is antiestrogenic in the uterus as well as the breast and mimics estradiol action on blood LDL cholesterol and bone. The distinct response profiles and their clinical significance are reviewed elsewhere (Witorsch 2000, 2002).

The mechanisms governing the nature of the biologic response to an ER ligand appear to be multifactorial. Aspects of these mechanisms involve: *a*) the particular ER isoform (α or β)

activated, *b*) ligand-induced conformation changes in the receptor, *c*) co-regulatory proteins that associate with the ligand-receptor complex, and *d*) the association between the ligand-ER complex and other transcription factors and genomic sites within the cell (Witorsch 2000, 2002). Chirality is another factor that influences the potency and/or nature of the biologic response to an ER ligand. Unlike symmetrical compounds, mirror images of asymmetric (or chiral) chemicals, referred to as enantiomers, differ from one another with regard to the orientation of their elements in space. Chirality also potentially impacts estrogenic activity; certain enantiomers have been shown to exhibit marked differences in ER binding and biologic activity (Witorsch 2000, 2002).

Anti-estrogenic effects may also occur by mechanisms other than those described above. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and certain PCBs that share chemical characteristics with this compound exhibit little binding to the ER but are ligands for another transcription factor, the Ah receptor (AhR). Anti-estrogenicity exhibited by these dioxin-like compounds involves cross-talk between the AhR signaling pathway and that of the ER. A complex resulting from activation of the AhR evokes an anti-estrogenic effect by interfering with the ability of ER-ligand complex to interact with the latter's genomic site (Safe 1998).

In summary, there are numerous reasons for inter- and intra-assay variation in estimating the estrogenic potency of environmental chemicals. These include *a*) the existence of several distinct molecular mechanisms of estrogen action, *b*) factors that distinguish *in vivo* from *in vitro* conditions (e.g., metabolic transformation of the ligand, pharmacokinetic and pharmacodynamic factors, and SHBG binding), *c*) the multitude of factors that confer tissue specificity of the direction of a ligand-induced response (estrogenic or anti-estrogenic), *d*) chirality of ligands, and *e*) cross-talk between signaling pathways. Because these factors are complex and not completely understood, it is difficult at this stage to choose appropriate assay conditions to standardize the potency of these substances for use in risk estimation.

Methods for Assessing Estrogenic Potency of Mixtures

Because sources of infant nutrition may contain a variety of estrogenic substances, assessing estrogenicity of infant formulas and endogenous and exogenous chemicals in human milk requires methods to address mixtures rather than single chemicals. Mixture assessments follow one of two basic approaches: Either the mixture is treated as a single substance, using dose-response data for the mixture as a whole (the so-called whole-mixture approach), or dose-response characteristics of the mixture are

predicted from data on the mixture components (the so-called component-based approach).

Assessing the whole mixture. Whole-mixture approaches obviate the need to assess interactions among individual mixture components and are therefore the most direct way to compare different mixtures (Feron et al. 1998; U.S. EPA 1986, 1988, 2000). The EDSTAC recommendations to screen specific mixtures of chemicals for endocrine activity (EDSTAC 1998) implied a whole-mixture approach.

The chief disadvantage of whole-mixture approaches is that a large number of different mixtures would need to be tested individually, because even small differences in mixture composition can affect toxicologic and dose-response characteristics, making it difficult to extrapolate data from one mixture to other mixtures. Without a way to extrapolate data from one mixture to a similar mixture, different infant nutrition sources would have to be treated as distinct, separate mixtures and estrogenic dose-response data generated and compared for each. Generating dose-response data for a whole mixture presents additional difficulties, because concentrating or diluting a mixture to produce a range of concentrations changes the dose-equivalent ratios of mixture components. Because mixture effects vary with both concentrations and ratios of the constituents (Berenbaum 1989; Borgert et al. 2001; Greco et al. 1995), an elaborate toxicologic assessment of individual whole mixtures is usually not practically or technically feasible.

Because of concerns about designing and interpreting mixtures studies, a joint committee of the Science Advisory Board and Science Advisory Panel (SAB/SAP) of the U.S. EPA recommended that screening and testing of mixtures for hormonal potential be delayed until the feasibility of such an approach could be assessed with the benefit of data on individual chemicals (SAB 1999). The technical limitations inherent in performing whole-mixture studies may also explain the paucity of toxicology data on the mixtures of exogenous chemicals that may be found in human milk or in other sources of infant nutrition. Despite those challenges, a few published mixture studies have potential relevance to sources of infant nutrition. A reconstituted mixture of PCB congeners similar to the profile of PCB congeners detected in human milk samples was more potent as an estrogen and as an antiandrogen in rats than Aroclor 1254, which is a technical-grade mixture of PCB congeners (Hany et al. 1999). The reconstituted mixture was effective in reducing testis weights and increasing uterine weights in rats exposed pre- and postnatally at concentrations 1–2 orders of magnitude greater than concentrations reported for human milk. It is unknown whether PCB mixtures could produce similar effects in

nursing infants at the concentrations reported for human milk. It also remains to be assessed whether those effects would be manifested in mixtures with other chemicals in human milk that might be estrogenic, antiandrogenic, or anti-estrogenic, such as endogenous hormones.

Assessing mixture components. The alternative method of assessing mixtures, the component approach, is to predict the toxicity of the whole mixture from toxicity data on the individual components of the mixture, including any known pharmacokinetic or pharmacodynamic interactions between those components. The chief advantage of this approach, at least in theory, is that the results can be used to predict the effects of various mixtures. One significant disadvantage is the technical challenge of designing and interpreting interaction studies for use in risk assessments (Borgert et al. 2001; U.S. EPA 1988). Failure to negotiate those technical challenges renders much of the literature on interactions inappropriate for use in mixture risk assessment (Borgert et al. 2001; U.S. EPA 1988).

The prototype model for component-based mixture risk assessment for chemicals with the same mechanism of toxicity is the TEF/TEQ approach (Safe 1990). Here, the toxicity of the whole mixture, expressed as TEQs, is estimated from the concentration of each component of the mixture multiplied by its toxic equivalency factor (TEF), i.e., its relative potency factor (Safe 1998). This approach assumes several toxicologic characteristics of mixture components, including *a*) that the individual chemicals all act through the same biologic or toxic pathway; *b*) that the effects of individual congeners in a mixture are dose additive at submaximal levels of exposure (i.e., that there is constant proportionality between the toxicity of the components such that their dose-response curves are parallel); and *c*) that the toxic manifestations of all congeners are identical over the relevant range of doses (Safe 1998).

Safe (1998) has enumerated the problems with applying the TEQ approach to environmental endocrine disruptors. Xenoestrogens are a structurally diverse set of chemicals and, as such, lack the similarities shared by true congeners. Uptake, distribution, and metabolism of xenoestrogens can vary considerably, making *in vitro* estimates of potency difficult to extrapolate to intact animals. The development of a TEF for estrogenicity is particularly hampered by three recent developments, as noted previously. A second form of the ER has been identified (Chang and Prins 1999; Gustafsson 2000; Kuiper et al. 1996), and the two forms of ER are activated differentially by some phytoestrogens and industrial chemicals (Kuiper et al. 1998); cross-talk occurs among signaling pathways for different hormones (Safe 1998), and both genomic and nongenomic pathways of

estrogen modulation are significant in estrogen-responsive cells (DuMond et al. 2001). Thus, estrogenic responses are a complex integration of cell signaling via two ER receptor subtypes, genomic and nongenomic pathways, and cross-talk with other hormone-signaling pathways (DuMond et al. 2001; Makela et al. 2000; Safe 1998) rather than deriving from a single biologic pathway as required for application of a TEF/TEQ approach.

Furthermore, the TEF/TEQ approach assumes a dose-additive model for mixtures of chemicals, but nonadditive interactions have been reported for some combinations of xenoestrogens. Both greater-than-additive (e.g., Arnold et al. 1997; Bergeron et al. 1994) and less-than-additive (McMurry and Dickerson 2001; Thorpe et al. 2001) effects have been reported, although there is some concern regarding the methods used to infer greater-than-additive effects (Borgert et al. 2001). Although few toxicologic interactions have been confirmed in animals exposed to concentrations of putative estrogenic chemicals reported in human milk, neither have dose-additive estrogenic effects been demonstrated. Before an estrogenic equivalence approach could be supported scientifically, dose additivity would need to be verified experimentally. Experimental verification would seem to be particularly important given the potential for exogenous chemicals to act by diverse hormonal mechanisms, as described previously in this paper.

One approach that has not, to our knowledge, been proposed in the literature is a non-interaction model for xenoestrogens based on statistical independence, sometimes called "response additivity." In such a model, each chemical behaves as if other chemicals in the mixture were not present (Bliss 1939; Finney 1971). Thus, independence models would predict no response when all mixture components are present at subthreshold concentrations. Independence models may need to be considered for exogenous estrogenic chemicals, because those chemicals may operate via independent estrogenic pathways at both the molecular and cellular level, as discussed in the previous section.

Of relevance to this discussion is a recent study by Payne et al. (2001) in which the *in vitro* estrogenic effects of a mixture of DDT and its metabolites (essentially, technical-grade DDT) could be modeled equally well by dose-additive and independence models at low concentrations. Because of the inherent variability of biologic responses, it may often be difficult to distinguish between these different no-interaction models at low chemical concentrations. Because testing metabolites of a chemical is similar to testing multiple doses of the parent compound, one would have expected dose-additive results (Finney 1971; U.S. EPA 2000). This example, therefore,

underscores the complexity by which chemicals can produce estrogenic responses, and it must be kept in mind that metabolites of a chemical may be estrogenic, but possibly via mechanisms different than that of the parent compound. A more recent *in vitro* study by the same group found dose-additive effects for a mixture of estrogen active chemicals (Silva et al. 2002). Nonetheless, a number of additional criteria remain to be fulfilled before a TEF approach could be considered for those chemicals, including correlating *in vitro* results with *in vivo* toxicity (Safe 1995, 1990; Safe et al. 1998).

Finally, the tissue-specific nature of some estrogenic responses further complicates assigning a single hormonal potency to a chemical. As alluded to earlier, the complexity of estrogenic responses is well illustrated by the drug tamoxifen, which can be antiestrogenic in breast tissue depending on the presence of endogenous estrogen and estrogenic in uterus, blood LDL cholesterol, and bone (Witorsch 2000, 2002). Other exogenous chemicals detected in human milk and in human adipose tissue may also have differential effects that are both dose and tissue dependent. Such effects could be mediated through agonistic activity at the AhR, through alteration of xenoestrogen metabolism, or through alteration of ER-activation kinetics as occurs with TCDD (Safe 1998; Safe et al. 1998), and via cross-talk with other cell-signaling pathways. In addition, TCDD has been reported to potentiate some systemic effects of some estrogens, including endogenous estrogen (Petroff et al. 2000, 2001). Because of the complexities inherent in discreetly categorizing the hormonal activity of exogenous chemicals, it seems that risk assessment methods based on relative potency estimates will be inapplicable for comparing risks of different infant nutrition sources.

Epidemiologic and Clinical Studies Relevant to Infant Nutrition

As described in this review, the state of the science does not provide a reliable methodology for predicting the relative estrogenicity of endogenous and exogenous chemicals in human milk or in infant formula. However, this does not preclude an assessment of estrogenic health effects from infant nutrition if other sources of data are available. In fact, epidemiologic and clinical data on infants exposed to different sources of nutrition are available and can potentially provide direct evidence of health effects. Though such studies are not typically sufficient to elucidate mechanisms underlying health outcomes in an exposed population, these may nonetheless be informative regarding adverse estrogenic effects in infants. Epidemiologic and clinical data regarding potential estrogenic effects in adults

are also available on persistent organic chemicals (for example, from the Seveso, Italy, accident) and phytoestrogens (e.g., Li et al. 1998) and could be informative as well. Carefully designed epidemiologic or clinical studies have the advantage of producing data directly from the population of interest and are therefore more readily interpretable to clinicians, public health officials, and ultimately to the public.

A number of epidemiologic studies have investigated risk factors for developing breast cancer. Because breast cancer risk may be affected by lifetime estrogen exposure, including exposures early in life, being breast-fed as an infant was among the factors evaluated in several of those studies (reviewed in Potischman and Troisi 1999). Four case-control studies reported a protective effect of having been breast-fed, whereas three others reported no association, except that one of the larger of the three studies found a protective effect for premenopausal breast cancer only. Exposure to human milk in infancy may be related to reduced risk of premenopausal breast cancer, but appears unimportant as a risk factor for postmenopausal disease. Although the mechanisms are unknown, the apparent protection observed in those studies may presumably arise from protective factors in the milk itself or from detrimental factors in formula preparations fed the comparison group (Potischman and Troisi 1999). Whether potential protective effects are related to estrogenic or antiestrogenic activity of human milk is at present unknown.

Clinical data currently available indicate a health benefit to offspring for 6 weeks of breast-feeding. At present, there is no evidence that infants breast-fed for more than 6 weeks suffer more adverse health effects, hormonally mediated or otherwise, than infants receiving infant formulas or other sources of nutrition. Effects similar to those observed following high-dose exposure to potent estrogens—e.g., *in utero* exposure to DES—to the best of our knowledge, have not been associated with breast-feeding or other sources of infant nutrition. Thus, the epidemiologic and clinical studies conducted to date suggest that breast-fed infants suffer no adverse estrogen-related health effects. Given that much of the data on breast-fed infants was collected several decades ago when levels of persistent contaminants in humans were likely higher than at present, particularly in countries such as the United States where the use/release of many of these chemicals has been banned or restricted (LaKind et al. 2001; Westphal 1986), estrogenic risks to infants from consumption of human milk should be considered *de minimis*. Nonetheless, the information is limited regarding hormone-related health effects associated with exogenous and endogenous hormonally active chemicals in human milk. Available literature on exogenous chemicals in human milk is complicated by the

natural variations of endogenous hormones in human milk.

Although clinical data are limited for infants fed soy-based formulas, no ill effects have been reported for these exposures other than soy-induced goiter, which was resolved in the 1960s with the introduction of iodine-supplemented infant soy formulas (Chorazy et al. 1995; Setchell et al. 1997; Whitten and Naftolin 1998). A small study associated soy-based infant formulas with autoimmune thyroid disease in children genetically predisposed to develop the condition (Fitzpatrick 1998; Fort et al. 1990). Essex (1996) notes that pediatricians and pediatric endocrinologists have not seen large numbers of infants with feminization—one theoretical outcome from high exposures to phytoestrogens such as isoflavones—and overtly estrogenic responses in infants consuming soy-based formulas have not been reported (Whitten et al. 1995). This is in contrast to reports of feminization of male infants whose mothers were taking oral contraceptives while nursing (Grosvenor et al. 1992), and breast enlargement in an 18-month-old female breast-fed infant whose mother had been using oral contraceptives (Madhavapeddi and Ramachandran 1985). A report of premature thelarche (early breast enlargement without additional signs of sexual development) in Puerto Rico noted that consumption of soy-based formula was one of several positive associations in 120 pairs of children (Freni-Titulaer et al. 1986); others included maternal history of ovarian cysts and consumption of various meat products.

To our knowledge, the only study on endocrinologic and reproductive outcomes in young adults fed soy-based infant formula is that of Strom et al. (2001), who conducted a retrospective cohort study comparing 248 adults fed soy-based formula as infants with 563 adults fed cow's milk formula as infants. Strom et al. (2001) found no statistically significant differences between these two groups (either men or women) for more than 30 outcomes, except for the following: Women fed soy-based formula had a slightly longer duration of menstrual bleeding (0.37 days) and greater discomfort (borderline significantly more common) associated with menstruation. Pubertal maturation, menstrual and reproductive history, height and weight, and general health were not different for the two groups. Because only limited data have been published on risks/benefits associated with these infant exposures in spite of the fact that infants consuming soy-based formulas have some of the highest exposures to estrogenic chemicals, Sheehan (1998) has advocated expanded, long-term research on populations of infants exposed to soy-based infant formulas. Epidemiologic data, such as data to be collected as part of the Study of Estrogen Activity and Development

(CODA 2001) sponsored by the National Institute of Environmental Health Sciences, may be useful in determining the extent to which various sources of infant nutrition may be associated with negative or positive health outcomes (Barrett 2002; Setchell et al. 1997; Sheehan 1998).

Although epidemiologic and clinical studies can potentially provide the most reliable data for comparing estrogenic risks of infant nutrition sources, such studies are themselves subject to several limitations. First, reliability depends on sufficient resolving power to detect low-frequency effects, and comparative risks are most accurate when all data are derived from large, well-designed studies. The quality and quantity of epidemiologic and clinical data are varied for sources of infant nutrition, especially for human milk containing environmental chemicals, making comparative risk assessment difficult. Ideally, infant health status and infant exposures to estrogenic compounds from all sources, including endogenous estrogens, should be assessed simultaneously if the data are to be used in comparative risk assessments. However, few studies include a credible assessment of both infant health status and infant exposures. For example, numerous problems have been identified in studies reporting on neurodevelopmental effects of prenatal and postnatal PCB exposure (Schell et al. 2001). Epidemiologic data often involve confounding exposures to mixtures that may not reflect the chemical mixture profiles typically found in the breast milk of lactating mothers.

The basis for epidemiologic and clinical approaches could be improved by simultaneously collecting data on health outcomes and levels of infant exposure to both xenoestrogens and, where applicable, to natural estrogens and phytoestrogens (Setchell et al. 1997) as are present in human milk and certain infant formulas. It will be equally important to measure concentrations of xenoestrogens in human milk as well as natural and nutritionally related fluctuations in levels of maternal hormones in human milk, including interindividual variations in human milk content (LaKind and Berlin 2002; LaKind et al. 2001; Safe 1998). It will be critical for such studies to be prospective and hypothesis driven rather than surveillance oriented. For example, the hypothesis that a specific component or mixture of components in human milk confers protection against premenopausal breast cancer deserves further investigation.

Conclusions

Comparative risk assessment of estrogenic risks from infant nutrition sources is not easily accomplished by toxicologic methods currently available. This is consistent with the conclusions of a recent Society of Toxicology expert panel that addressed challenges to assessing the

toxicity of low-level environmental chemical mixtures (Nilsson 2000; Teuschler et al. 2002). Health risk estimates and public health recommendations should not be based on estimates of estrogenic potency or estrogenic effects at this time, because of the tremendous uncertainties inherent in data interpretation and the very real possibility of introducing rather than mitigating perceived health risks to infants. Equating the results of *in vitro* tests (such as binding of anthropogenic compounds to a hormone receptor) to toxic effects on human endocrine systems is a speculative approach; health end points should be considered in any evaluation of risk (Nilsson 2000). In addition, tests developed to determine whether a chemical is an endocrine disruptor should be able to distinguish between chemicals that cause changes in the endocrine system, i.e., endocrine active, from those that cause harmful effects, i.e., endocrine disruptive.

Mechanistic approaches have been used to improve the pharmacotherapy of many diseases and hold great promise for advancing human health risk assessment as well. However, until the complex mechanisms of estrogen action are clearly understood in relationship to specific adverse health effects, methods that evaluate total health outcomes and risks (such as epidemiologic and clinical studies) would seem to provide more useful information regarding the potential risks to offspring of endocrine-disruptive chemicals in breast milk or infant formulas. To date, the available epidemiologic data do not suggest an increased incidence of any estrogen-related adverse effect in either breast-fed or formula-fed infants. Although epidemiologic approaches suffer a number of limitations, large populations are available for further study using standardized methods.

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